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The Exploitation of Nematode-Responsive Plant Genes in Novel Nematode Control Methods*

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Abstract: The molecular interactions between plants and sedentary nematodes are undergoing intense study, not only for reasons of fundamental research but also for the potential benefits to agriculture. The present technology allows the transformation of an increasing number of crop plants, providing new ways to introduce resistance against plant-parasitic nematodes. The ability of sedentary nematodes to induce specialized feeding sites in plant roots is one of the most fascinating aspects of this host–parasite interaction. Molecular approaches have been initiated to identify and characterize plant genes altered in expression after infection by sedentary nematodes. The results obtained indicate that many genes indeed become up-regulated upon nematode infection. Surprisingly, several so-called constitutive promoters that are normally used to achieve high expression in plant cells are completely 'silenced' in the feeding sites within days after nematode infection. Generally, there are two options available for the genetic engineering of nematode resistance: the synthesis of anti-nematode proteins or the localized production of a cytotoxic protein that interferes with the development of feeding cells. Nematode-induced promoters are very useful for the production by plants of sufficiently high levels of anti-nematode proteins at feeding sites. Alternatively, interfering with feeding-cell development is somewhat similar to the hypersensitive response evoked by nematodes in a naturally resistant plant. Here, destruction of specific plant cells can be achieved by the localized expression of a cytotoxin such as barnase, a potent ribonuclease. This approach, however, calls for a highly specific 'non-leaky' promoter, which is active only in the feeding cells. Another possibility is to use a two-component system, where the leakiness of the promoter in other tissues is counterbalanced by the constitutive expression of a neutralizing gene.

Key words: barnase, β-glucuronidase, *Lea* gene, promoter trapping, proteinase inhibitor, sedentary nematodes.

1 INTRODUCTION

Plant-parasitic nematodes are amongst the most damaging pests in world agriculture. In particular, the sedentary endoparasitic genera *Meloidogyne*, *Globodera* and *Heterodera* are responsible for major crop losses.[1] These nematodes establish and maintain an intimate relationship with their host.[2] After invading the plant root, they migrate to the vascular cylinder in search of a cell which can serve as an initial feeding cell. In response to repeated stimulation by the parasite, probably caused by salivary secretions, a series of dramatic cellular changes is triggered in and around the initial feeding cell. Depending on the nematode species, this cell either develops into a syncytium (for cyst nematodes such as *Globodera* and *Heterodera* spp.), or several cells are stimulated to form a system of giant cells (with the root-knot nematodes *Meloidogyne* spp.). Unlike migratory endoparasitic nematodes that kill the cells from which they feed, it is essential for sedentary endoparasites that the feeding cells remain healthy and metabolically active throughout the nematode's life cycle.

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Control of plant-parasitic nematodes is currently based on the three principal approaches: crop rotation, use of agrochemicals and host resistance. When available, the use of resistant crop cultivars[3] is undoubtedly the most economically and environmentally preferred method. However, the use of natural resistance is limited for a variety of reasons, most importantly because plant resistance is often restricted to specific species or races of a particular nematode genus. Plant biotechnology, however, now offers opportunities to design novel types of resistance constructs and to introduce these into any plant crop, providing effective and durable ways of nematode control.

2 SOME NEW METHODS OF NEMATODE CONTROL

2.1 Analysis of feeding site formation

A combination of light and electron microscopy studies of fixed samples, as well as time-lapse video microscopy of living material, has provided insight into the establishment and structure of the nematode feeding site.[2,4,5] The syncytium develops by the breakdown of plant cell walls and subsequent fusion of neighbouring cells, whereas a giant cell is generated as the result of repeated nuclear divisions without cytokinesis. Although these feeding cells (syncytia and giant cells) differ from each other in ontogenesis and structure, they both function as transfer cells supplying the nematode with sufficient nutrients for growth and reproduction. This common function is reflected in an analogous ultrastructure. Syncytia and giant cells are both hypertrophied and multinucleated, with a dense granular cytoplasm containing an increased amount of rough endoplasmic reticulum and a larger number of mitochondria than normal cells. The nuclei are enlarged and lobate, the central vacuole is greatly reduced in size, and invaginated cell walls indicate extensive solute exchange with the vascular system. Root-knot nematode development is further accompanied by the proliferation of nearby pericycle and cortical cells, resulting in the characteristic root-knot or gall.

The substantial changes that occur to plant cell structure and metabolism at the nematode feeding site indicate that plant gene expression must be re-programmed in the affected cells. An excellent and easy way to study gene expression is the use of promoter fusions to the β-glucuronidase (*uidA* or *gus*) gene.[6] GUS is an enzyme that specifically cleaves β-glucuronide bounds, regardless of the nature of the other moiety. Substrates have been made that release either a coloured product, a fluorescent product, or a precipitative stain upon cleavage. The last type of histochemical substrate is ideal for the study of cellular gene expression patterns. A blue precipitate forms only in those cells in which the GUS enzyme is expressed. After GUS assay, plants containing the *gus* gene fused to the promoter from a gene expressed in the infection sites will show blue infection sites in the roots. Because the test is so easily performed, many infected plants can be analyzed simultaneously and in all organs, for example to compare different time points after infection or to analyze the effect of different nematode species.

Several plant genes that could be expected to play a role in the establishment or functioning of the feeding sites have already been cloned and promoter-*gus* fusions, transformed into plants, were readily available for study after nematode inoculation. In this way, expression of a gene encoding the structural cell wall protein extensin has been studied during the early phases of infection in tobacco.[7] Extensin is induced in actively dividing cells[8] and following attack by compatible or incompatible pathogens.[9] The tobacco cyst nematode induces extensin expression only weakly, probably due to wounding during penetration and migration through the roots. In contrast, high extensin expression is observed in galls induced by the root-knot nematode.

Another example of an up-regulated gene is given by Cramer,[10] who has shown that the promoter of the gene encoding hydroxymethylglutaryl CoA reductase (HMGR) is activated shortly after the onset of feeding by the root-knot nematode, and high levels of activity are seen throughout the gall one week after nematode inoculation.[11] HMGR is a key enzyme for sterol synthesis, a group of compounds that cannot be synthesized by the nematode[12] and for which it is entirely dependent on its host.

A tobacco gene (*TobRB7*) encoding a membrane protein believed to function as a water channel, has recently been shown to be up-regulated in giant cells induced by different species of *Meloidogyne*.[13] A 1·8-kb *TobRB7* promoter fragment was fused to *gus*, transferred to tobacco and the resulting transgenic plants were infected by *Meloidogyne incognita* (Kofoid and White) Chitwood. A strong increase in GUS activity was observed inside the giant cells compared to other root tissues. Promoter deletion studies showed that the root-specific expression could be uncoupled from nematode-induced expression. A 300-bp promoter fragment still conferred high expression inside the giant cells, but no GUS activity could be detected elsewhere in the plant. The protein encoded by the *TobRB7* gene might be important in regulating the water status of giant cells. Interestingly, the *TobRB7* promoter is not activated in syncytia induced by the tobacco cyst nematode.

The availability of cloned genes involved in the regulation of the cell cycle has allowed a molecular study of the cell cycle in the early phases of feeding cell formation.[14] Using transgenic *Arabidopsis thaliana* (L.)

Heynh. plants containing the *cdc2a* promoter–*gus* construct, GUS staining was observed shortly after syncytium initiation by the cyst nematode *Heterodera schachtii* (Schmidt). Very intense GUS staining is seen in feeding cells induced by either cyst or root-knot nematodes during the first days of feeding cell development, while at later stages GUS activity is much weaker or absent. Analogous results have been obtained with *cyc1At*, a mitotic cyclin gene from *A. thaliana*, although the *gus* expression is much more transient in this case. During the early phases of feeding site formation, root-knot nematodes induce endomitosis in the feeding cells, as well as cell proliferation in the surrounding tissues. Thus, it is not unexpected that we see the induction of two cell cycle genes both in nematode-induced giant cells and in galls. In contrast, no clear mitotes have ever been observed in cyst nematode-induced feeding cells.

Although more detailed studies are needed to understand the molecular changes occurring in the feeding cells, the first results already indicate that certain widely accepted hypotheses have to be treated with caution. For example, it is generally believed that giant cells and syncytia differ in their ontogeny, yet the early induction of cell cycle genes in both systems clearly points to some similarities.[14] Likewise, although the final functions of the different types of feeding cells are considered to be analogous, the membrane channel protein gene is induced only in giant cells and not in syncytia.[13] Another unexpected result from the study of promoter–*gus* fusions is that several of the most powerful promoters that are presently available for gene expression in plant roots, such as the CaMV 35S, are completely silenced within days after nematode infection.[15] Down-regulation begins immediately after the induction of the feeding cells, so that these so-called 'constitutive' promoters are not a very useful tool to engineer the expression of a protein at nematode infection sites.

Besides for the detailed expression analysis of known cloned genes, plant–*gus* fusions can also be made *in vivo* at random and then screened for specific expression patterns. This 'promoter trapping' or 'tagging' approach is based on the random integration of a promoterless *gus* gene after transformation into *A. thaliana*.[16] When inserted downstream from a plant promoter that is inducible by nematode infection, it is expected that a higher GUS activity will be seen at the infection site. The elegance of this method lies in its ability to visualize directly induced expression in nematode feeding sites at various stages of the interaction, while analyzing the specificity of expression using uninfected parts of the same plant and control plants. With this approach, three lines have been isolated that show high GUS staining inside syncytia induced by *H. schachtii*,[15] whereas in control roots, GUS staining is restricted to the vascular cylinder. In this laboratory, among several lines with induced expression, two have been identified that show high GUS staining almost exclusively inside *M. incognita*- or *H. schachtii*-induced feeding cells. In control plants, by comparison, staining is very low and restricted to the base of lateral roots (Barthels, N., Gheysen, G. & Karimi, M., unpublished). This 'tagging' approach is being used currently in several laboratories and the analysis of more than 1000 transgenic lines indicates that approximately 4% of the tagged genes are up-regulated at nematode infection sites (Barthels, N., Gheysen, G. & Karimi, M., unpublished; Sijmons, P., Grundler, F. & Puzio, P., 1995, pers. comm.).

Plant genes that are up-regulated after nematode infection have also been isolated by differential screening of cDNA libraries or from subtraction cDNA libraries, for example a potato catalase,[17] the *E2* gene of the protein ubiquitination pathway[18] and a *Lea*-like gene from tomato.[19]

2.2 *Lea*-related genes are differentially expressed at nematode feeding sites

The *Lea*-like gene, named *Lemmi9* (acronym for *Lycopersicon esculentum* cv. Marmande *Meloidogyne incognita*), was identified by differential screening of a cDNA library from tomato galls induced by *M. incognita*. As shown by hybridization *in situ*, this gene is locally and highly induced in the giant cells at the later stages of nematode infection. *Lemmi9* shows significant sequence similarity (70% amino acid identity in exon 2) with the cotton gene *Lea14-A* encoding a late-embryogenesis-abundant (LEA) protein.[20] *Lea* genes are predominantly expressed during late embryogenesis, where the encoded proteins are probably important in protecting the embryo during desiccation of the seed. The *Lemmi9* protein could, therefore, be acting as an osmoprotectant in giant cells. In contrast to most *Lea* genes, *Lemmi9* is not induced in water-stressed plants or by abscisic acid treatment.[19]

To evaluate whether other *Lea* genes might also be induced in root-knot nematode-induced giant cells, a *Lea14-A-gus* transgenic tobacco line has been analyzed. Seeds from this line were surface sterilized and the resulting seedlings then infected *in vitro* with *M. incognita* (for a more detailed description of the method, see Ref. 7). Infection sites were harvested at one-week intervals (from one to four weeks) and assayed for GUS activity. The temporal expression pattern looked very similar to that obtained by hybridization *in situ* for *Lemmi9*, i.e. no GUS-staining at early stages, increasing GUS activity during the development of the nematode and a maximum staining at four to five weeks after infection. However, when the galls were sectioned to analyze the cellular expression pattern, it turned out that the *Lea14-A* promoter is not activated at all in giant cells. Instead, very high promoter activity is evident around the developing egg mass (Fig. 1).

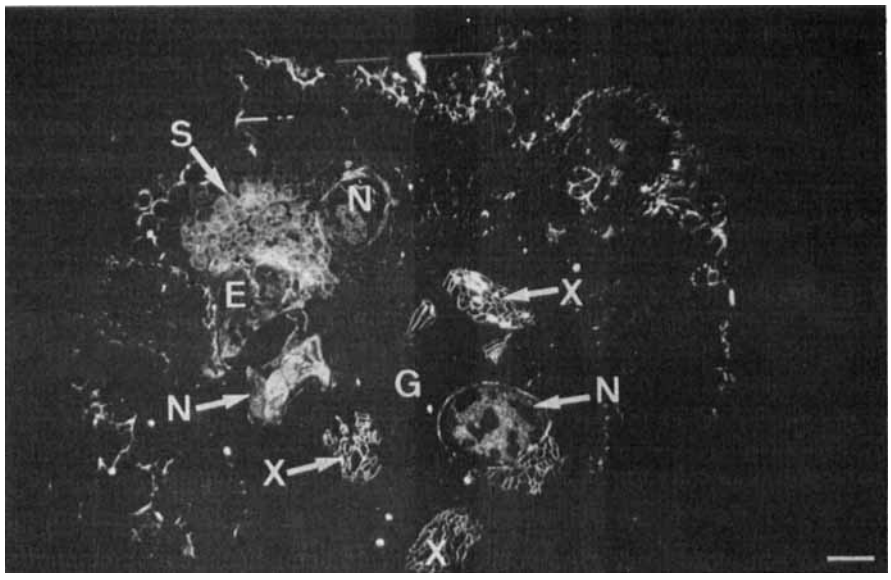

Fig. 1. Dark-field micrograph of a GUS assay on tobacco plants containing the *Lea14-A-gus* gene, four weeks after infection with *Meloidogyne incognita*. After the GUS assay, the roots have been fixed, embedded and sectioned. The figure shows a section through a gall. The GUS staining is only present close to the egg mass of the nematode. It is clearly visible that the plant root cells expressing the *gus* gene are separating from one another. This is most probably caused by enzymes secreted from the egg mass, which facilitates the extrusion of the eggs from the gall into the soil. Abbreviations: E, egg mass; G, giant cells; N, nematode; S, GUS staining (red in colour picture; available upon request); X, xylem vessels. Bar = 0·2 mm.

Clearly, this induction is correlated with maceration of the plant root tissue. This cell loosening, due to the release of enzymes by the nematode, is necessary for the extrusion of eggs from the root into the soil. Since *Lea14-A* is wound-inducible (Galau, G. & Hughes, W., 1995, pers. comm.), activation of the *Lea14-A* promoter in galls can, therefore, be explained as a reaction to tissue damage.

2.3 Nematode-responsive plant promoters as tools for engineered resistance

Genetic engineering is becoming a routine technology for many crop plants and so offers novel perspectives to introduce nematode resistance. There are already numerous examples of transgenic crop cultivars genetically modified to be resistant against viruses or insects.[21,22] The most widely used approach is to express constitutively a protein that interferes with the development of the pathogen or the pest. Such constitutive expression, however, has some serious drawbacks, not least of which are the legal and social aspects of the presence of the protein in consumed plant parts.

As described above, fundamental research has identified several plant promoters that are either up- or down-regulated upon nematode infection. Depending on the strategy, a promoter can now be chosen with the desired spatial and temporal expression pattern for producing a protein that is inhibitory to one of the steps in pathogenesis. For sedentary endoparasites, on which this review concentrates, two main approaches can be envisaged: either the nematode is attacked directly by expressing a nematoxic protein, or it is affected indirectly by destroying the feeding cells or inhibiting the functioning of the feeding cells. There is now evidence that both approaches are feasible.

2.4 Anti-nematode strategies

The nematode can be attacked at many different stages in its life cycle, and some of the strategies described here are also applicable to migratory endoparasites. For example, (i) a root-specific promoter could be used to produce a protein interfering with nematode attraction to the plant or with its orientation and migration inside the roots; (ii) for migratory endoparasites and cyst nematodes, causing substantial necrosis during migration, a more specific response could be obtained by using a wound-inducible promoter;[23] (iii) feeding-cell-specific promoters can be used to produce proteins that are inhibitory to the nematode during the sedentary stage or (iv) the root-knot nematode egg mass could be targeted by using promoters such as that obtained from the *Lea14-A* gene. These approaches are discussed in the next paragraphs.

(i) Nematode sensory perception is still very poorly understood, but a better knowledge would possibly provide a basis for the design of plants producing inhibitory antibodies. The feasibility of expressing functional antibodies against pathogens in plants has been demonstrated recently[24] in that transgenic plants expressing a single-chain antibody against the artichoke mottled

crinkle virus caused reduction of infection incidence and a delay in symptom development.

(ii) The cuticle, the nematode's outer layer that also lines the oesophagus and intestine[25] can be damaged by specific proteins. For example, collagen is one of the major structural proteins of the cuticle and during moulting the nematode produces collagenases to shed the old cuticle. Plants do not naturally contain collagen and, therefore, engineered production of these collagenases by the plant could destroy the integrity of the cuticle. It is very unlikely that nematodes would be able to evolve resistance to collagenase, since any mechanism to destroy collagenase (or that resulted in a decreased sensitivity to it) would interfere with the normal moulting pattern. Other candidates for weakening the cuticle are proteins produced by the nematodes' natural enemies such as predatory fungi.

(iii) Nematode feeding is an interesting target and could be inhibited, for example, by blocking feeding cell development (see further), using monoclonal antibodies against the nematode saliva,[26] or by interaction with the digestive system (e.g. proteinase inhibitors).

(iv) Finally, several egg-parasitizing fungi are known to produce proteins that damage the nematode egg-shell (e.g. a subtilisin protease produced by *Verticillium chlamydosporium* Goddard[27]).

The most advanced anti-nematode strategy to date is based on proteinase inhibitors. These are proteins, often present in plant seeds, that interfere with digestive enzymes. Transgenic plants harbouring the cowpea trypsin inhibitor gene fused to a constitutive promoter show only retarded development of nematodes[11] or a smaller proportion of females.[28] Probably, the local down-regulation of the 'constitutive' promoter exactly where its activity is needed, the feeding site, hinders production of effective levels of the proteinase inhibitor. Not only the use of an up-regulated promoter, but also of proteinase inhibitors with the correct specificity for the nematode proteinases,[29] could improve this system dramatically.[30]

Most of these anti-nematode proteins should have little or no effect on plant cells, so expression need not be confined exclusively to the feeding cells. Possible drawbacks to such an approach might be the development of virulent pathotypes overcoming the effect of the anti-nematode proteins (unlikely for collagenase) or, in some cases, a specificity that is limited to certain nematode species.

2.5 Anti-feeding site strategies

The second approach, interfering with the development of the feeding cells, has the advantage that the nematode has no genetic influence over the possible degeneration of the feeding structure. Indeed, the basic concept here is that promoters from genes necessary for a compatible interaction are used against the nematodes. If the nematode loses its ability to induce these engineered genes, then this would result in failure to induce a feeding site.

Even a slight decrease in the efficiency of the food supply might be sufficient to reduce nematode multiplication or suppress the formation of females. Blocking the expression of plant genes that are normally up-regulated and important for the induction or maintenance of the feeding site should be feasible by using the antisense strategy[31] and a promoter which is highly induced, but not necessarily uniquely expressed inside the feeding cells, would be a good candidate for this task. As described above, several genes with this type of expression pattern have been identified.

Highly specific, non-leaky, expression in the feeding cells is needed, however, if a gene for a cytotoxic protein is introduced into the plant. Very potent cytotoxic genes have been isolated, e.g. the RNAse barnase. Engineered, exclusive expression of barnase in the tapetum cells of plant flowers causes the complete destruction of this cell layer, resulting in male sterile flowers.[32] As it is very unlikely that plants have evolved genes that are expressed uniquely in nematode-induced feeding cells, one can either try to dissect out a nematode-responsive element in induced promoters or counterbalance the leaky expression of the promoter in other organs of the plant (two-component system). As mentioned above, Opperman *et al.*[13] could indeed identify a giant cell-specific plant promoter fragment. An example of a two-component system is the combination of barnase and its inhibitor barstar:[33] the cytotoxic barnase is expressed using a feeding cell-induced promoter, whereas the neutralizing gene—encoding barstar—is expressed using a 'constitutive' promoter that is down-regulated in the feeding cells. In this way, all plant organs, except the feeding cells, are protected against any leaky expression of barnase. During induction, the concentration of the cytotoxin will increase locally in the feeding cells while the level of the inhibitor decreases in the same cells. Eventually, a threshold level will be reached in the feeding cell where the barnase can no longer be neutralized, leading to the degeneration of the feeding cell and starvation of the parasite. This type of engineered resistance would mimic the natural hypersensitive response generated in an incompatible plant/nematode interaction. The important advantage of the two-component system is that it has an extra safety feature built in for any leaky expression of the feeding-cell-specific promoter in other organs of the plant or under different circumstances.

3 CONCLUSION

Our knowledge of the molecular events occurring during plant–nematode interactions is still very limited.

Nevertheless, the plant genes that have been identified as being responsive to nematode infection are not only giving insight into the infection process, but are also providing promoters that are very useful for the design of novel resistance strategies. The prospect of having available in the near future a variety of engineered resistance genes to be tested in many different plants offers great promise for the control of major nematode pests in an effective, environmentally sound and inexpensive manner.

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